Defective Mer Receptor Tyrosine Kinase Signaling in Bone Marrow Cells Promotes Apoptotic Cell Accumulation and Accelerates Atherosclerosis

Hafid Ait-Oufella, Vahid Pouresmail, Tabassome Simon, Olivier Blanc-Brude, Kiyoka Kinugawa, Régine Merval, Georges Offenstadt, Guy Lesèche, Philip L. Cohen, Alain Tedgui, Ziad Mallat

Objective—To study the role of Mer receptor tyrosine kinase (mertk) in atherosclerosis.

Methods and Results—We irradiated and reconstituted atherosclerosis-susceptible C57Bl/6 low-density lipoprotein receptor-deficient female mice (ldlr−/−) with either a mertk+/+ or mertk−/− (tyrosine kinase-defective mertk) bone marrow. The mice were put on high-fat diet for either 8 or 15 weeks. Mertk deficiency led to increased accumulation of apoptotic cells within the lesions, promoted a proinflammatory immune response, and accelerated lesion development.


Key Words: apoptosis ■ atherosclerosis ■ phagocytosis ■ inflammation

Accumulation of apoptotic cells and debris within the lipid core is a major feature of advanced human atherosclerotic lesions and has been associated with increased susceptibility to thrombotic plaque complication.1,2 Accumulation of apoptotic material may result from increased susceptibility of vascular smooth muscle cells3 or macrophages4 to apoptosis, or most likely, from defective clearance of apoptotic material from the lesions,5 attributable, at least in part, to competition between apoptotic bodies and oxidized phospholipids of the lipid core6,7 (reviewed in8). Reduced phagocytic removal of apoptotic cells may play a major role in the perpetuation of the inflammatory response in atherosclerosis,8 by hampering the potent immunosuppressive response that follows the ingestion of apoptotic bodies9,10 or by directly promoting inflammatory responses attributable to the high concentration of proinflammatory oxidized phospholipids on apoptotic cell membranes.11,12 Thus, unraveling the cellular and molecular mechanisms involved in the clearance of apoptotic cells from atherosclerotic lesions is of particular importance to our understanding of the pathophysiology of atherosclerosis.8 A large variety of potentially distinct pathways are involved in the clearance of apoptotic cells under physiological conditions,10 but the contribution of each of these pathways to the disposal of dying cells in disease states is still largely unknown. We have recently shown that milk fat globule EGF-like Factor 8 (Mfge8), a glycoprotein involved in phagocytic clearance of apoptotic cells, was required for efficient removal of apoptotic cells during both early and advanced atherosclerosis13 and promoted an athero-protective regulatory T cell immune response.13 Others have recently identified a role for C1q-mediated clearance in containing the size and complexity of early lesions, without altering the development of more advanced plaques.14 Deficiency in leukocyte transglutaminase-2 was also associated with a trend toward higher accumulation of apoptotic material in atherosclerotic lesions.15 Thus, distinct disposal pathways may play distinct and specific roles in atherosclerosis. In the present study, we hypothesized that Mer receptor tyrosine kinase (mertk), a receptor expressed in bone marrow–derived macrophages and involved in apoptotic cell clearance,16,17 may significantly inhibit apoptotic cell accumulation within atherosclerotic lesions and alter lesion development and progression.

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As shown in Figure 1a and supplemental Figure I (available online at http://atvb.ahajournals.org), mertk was expressed by macrophages of human atherosclerotic lesions. Smooth muscle cells showed no mertk expression (supplemental Figure I). We also detected mertk mRNA in murine atherosclerotic lesions (data not shown). To address the direct role of macrophage mertk expression in atherosclerosis, we first studied ldlr−/−/mertk−/− and ldlr−/−/mertk+/− chimeric mice after 8 weeks of high-fat diet (see supplemental Methods). As shown in Figure 1,
we found extensive accumulation of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells in the lesions of mertk−/− mice, whereas TUNEL positivity was barely detectable in the lesions of mertk+/+ mice. TUNEL positivity occurred within the lipid core (Figure 1), an area rich in macrophages. Consistent with this observation, lesions of mertk−/− mice showed a marked increase in the acellular core area localized around TUNEL-positive nuclei, compared with lesions of mertk+/+ mice (Figure 1). Interestingly, this was associated with a marked 66% increase in lesion size in mertk−/− compared to mertk+/+ group (Figure 1), despite similar total cholesterol level (supplemental Table I). The increase in lesion development was still significant after 15 weeks of high-fat diet (Figure 1). It is noteworthy that increased accumulation of apoptotic cells in lesions of mertk−/− mice was not attributable to acceleration of lesion development because large lesions of wild-type mice (15 weeks of high-fat diet) consistently showed lower levels of apoptotic cell accumulation compared to smaller lesions of mertk−/− mice (8 weeks of high-fat diet). In addition, increased accumulation of apoptotic debris in lesions of mertk−/− mice could not be attributed to increased susceptibility of mertk−/− macrophages to apoptotic death (supplemental Figure II).

We next assessed lesion composition. Smooth muscle cell accumulation was very limited (data not shown). Percent macrophage area was similar between mertk−/− and mertk+/+ groups. However, absolute lesion area occupied by macrophages (MOMA2 staining) was significantly higher in mertk−/− group compared with mertk+/+ group (Figure 2), which was associated with increased accumulation of T cells (supplemental Figure III).

These results suggest that acceleration of atherosclerosis in mertk−/− mice was related to enhanced plaque inflammation. Collagen accumulation as assessed by Sirius red staining was not different between mertk−/− and mertk+/+ groups (supplemental Table I). However, it is interesting to note that lesion progression in mertk+/+ mice (between 8 weeks and 15 weeks of diet) was associated with increased accumulation of collagen (from 20% to 38%, supplemental Table I), whereas lesion progression in mertk−/− group occurred without any change in the relative collagen content (from 25% to 26%, supplemental Table I), leading to an imbalance between macrophage and collagen accumulation within the lesions. These results clearly suggest that lesion development and progression in mertk−/− mice is associated with increased accumulation of apoptotic bodies and is driven by an inflammatory process, hampering lesion repair.

Mutant mice that lack the related receptor tyrosine kinases, Tyro 3, Axl, and Mer (TAM), develop autoimmunity resulting from the hyperactivation of antigen-presenting cells,18 suggesting an important role of TAM receptors in the control of innate immunity. In addition, cytokine-dependent activation of TAM receptors inhibits the innate immune response through induction of SOCS1 and SOCS3.19 Thus, even though mertk may activate antinflammatory pathways in response to apoptotic cell clearance,20 downmodulation of NF-κB transcriptional activation may involve separate signaling pathways.21 To assess the inflammatory profile, we examined cytokine production by cultured splenocytes and purified CD4+ T cells. Defective mertk signaling was associated with a reduction in antiatherogenic...
interleukin (IL)-10 production by splenocytes and purified CD4+ T cells, and a clear switch toward a proinflammatory and proatherogenic Th1-related phenotype, as shown by enhanced production of tumor necrosis factor (TNF)-α, IL-12, and IFN-γ (Figure 2).

In summary, we show that defective mertk signaling enhances accumulation of apoptotic cells, induces a marked inflammatory phenotype, and accelerates lesion development. Our results are in line and extend previous ones from our group and others showing acceleration of atherosclerosis in association with defective apoptotic cell clearance.13–15 Taken together, these studies underscore the critical role of efficient apoptotic cell clearance in limiting the inflammatory response during the development and progression of atherosclerosis. The results may be relevant to the understanding of the mechanisms of lesion acceleration in diseases characterized by defective apoptotic cell clearance such as systemic lupus erythematosus.

Acknowledgments

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Disclosures

None.

References


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Supplementary Figure 1 online. a) Mertk expression (red, arrows) in macrophages at the margin of the necrotic core of a human atherosclerotic plaque. Arrowheads point to a layer of macrophages showing no mertk expression. b) Staining for α-actin (green) in the fibrous cap of a human atherosclerotic plaque. c) Incubation of the same section with anti-mertk antibody yielded no staining indicating absence of mertk expression in plaque smooth muscle cells.
Supplementary Figure 2 online. Bone marrow-derived macrophages were prepared from *Mertk*^{+/+} and *Mertk*^{-/-} mice and stimulated in vitro with either staurosporine or C2-ceramide in order to induce apoptotic cell death. Results are from 3 mice/group.
Supplementary Figure 3 online. CD3 (red) staining in lesions of Ldlr–/– reconstituted with either Mertk+/+ or Mertk–/– bone marrow and fed atherogenic diet for 15 weeks. Increased infiltration of CD3 positive lymphocyte was found in Mertk–/– mice.
Table 1 online. Weights, serum cholesterol levels and collagen content in the aortic sinus of

\(ldlr^{+-}/mertk^{+-}\) and \(ldlr^{+-}/mertk^{++}\) chimeric mice.

<table>
<thead>
<tr>
<th></th>
<th>(ldlr^{+-}/mertk^{++})</th>
<th>(ldlr^{+-}/mertk^{+-})</th>
<th>(ldlr^{+-}/mertk^{++})</th>
<th>(ldlr^{+-}/mertk^{+-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 weeks, n=5</td>
<td>21.9±1.1</td>
<td>20.8±0.4</td>
<td>21.6±1.3</td>
<td>19.4±0.8</td>
</tr>
<tr>
<td>15 weeks, n=4</td>
<td>21.6±1.3</td>
<td>19.4±0.8</td>
<td>7.3±0.3</td>
<td>7.1±0.1</td>
</tr>
<tr>
<td>15 weeks, n=5</td>
<td>24.8±3.3</td>
<td>38.0±5.9</td>
<td>26.0±4.1</td>
<td>7.6±0.3</td>
</tr>
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Values are means ± s.e.m.
Supplementary Methods online:

We used a bone marrow reconstitution model to obtain the development of fatty streaks in which macrophages express a tyrosine kinase-deficient mertk, leading to defective mertk signaling\(^1,^2\). For this purpose, we irradiated and reconstituted atherosclerosis-susceptible C57Bl/6 low-density lipoprotein receptor deficient female mice (\(ldlr^{-/-}\)) with either a mertk\(^{+/+}\) or mertk\(^{-/-}\) (tyrosine kinase-defective mertk) bone marrow. Ldlr-deficient mice were preferred to Apoe-deficient mice because Apoe per se contributes to apoptotic cell clearance\(^3\). All mice were on a pure C57Bl/6 background. After 4 weeks of recovery, the chimeric mice were put on atherogenic diet (15% cacao butter, 1.25% cholesterol without cholate) to induce lesion development. All experiments were performed in accordance with the European Community Standards on the Care and Use of Laboratory Animals. The heart was fixed immediately after sacrifice. Successive 10-\(\mu\)m transversal sections of aortic sinus were obtained and stained with Oil red O, Sirius red or using a monoclonal rat anti-mouse macrophage antibody (clone MOMA-2, MAB1852; Chemicon), as previously described\(^4\). TUNEL staining was performed using a stringent technique on fixed cryo-sections avoiding the use of proteinase K. Human atherosclerotic plaques were obtained from endarterectomy specimens and were previously described\(^5\). Staining for mertk was performed using the rabbit anti-human mertk antibody from Abcam. Smooth muscle cells were detected using the 1A4 clone from Dako. Macrophages were detected using an anti-CD68 monoclonal antibody from Dako. Computerized quantifications were performed using Histolab software. At least 4 sections per mouse were examined for each immunostaining and appropriate negative controls were performed. Statistical analysis was performed using the Mann-Whitney test.

References:


